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Method Validation Approaches for Pharmaceutical Assessments – Highlights with High Performance Thin Layer Chromatographic (HPTLC) Techniques

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Additional information is available at the end of the chapter

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Abstract

Method validation is an important activity for pharmaceutical evaluations to ensure that analytical methods are suitable for their intended use. With particular focus on active ingredient and impurities, the implementation of different categories of method validation are explained for qualitative and quantitative methods. Detailed explanations with example approaches are provided for the key aspects of method validation, namely specificity, accuracy, linearity, limits of detection/quantitation, precision, robustness, and method range. While all of the sections outlined for method validation are generally applicable for a variety of techniques commonly used in pharmaceutical analysis (i.e., UV and HPLC instrumentation), focused attention is provided for examples that have been implemented using high performance thin layer chromatographic techniques.

Keywords: method validation, pharmaceuticals, HPTLC, assay, active pharmaceutical ingredient, impurities

1. Introduction

Method Validation (MV) is a development process undertaken to establish, within acceptable statistical bounds, that an assessment procedure or method consistently yields a “true” result both in “within laboratory” and “among laboratories” testing. Pharmaceutical product quality assessments are focused on methods for the active pharmaceutical ingredient (API) and related impurities. Being able to perform methods of analysis to assess product quality is critical in law enforcement and regulating commerce. In addition, for new drug products, these quality determinations are surrogate performance indicators for assuring the safety and efficacy of a

pharmaceutical product. The safety and efficacy of a pharmaceutical product are established with a “pivotal lot” production of the product and the characterization of this lot with well validated methods with acceptable performance characteristics is critical to assure that future production lots have the same quality characteristics as the “pivotal lot”, thereby assuring they have equivalent safety and efficacy.

In the United States of America (USA), there are both private and public standards; the private standards are created through a USA Food and Drug Administration (FDA) approval process of industry method submissions that can be used for law enforcement, and public standards, which are promulgated in the monographs of the United States Pharmacopeia (USP) [1], that may be used in law enforcement or to support commercial agreements. The private standards, which are not publicly available, are private agreements between the approving government body and the submitting industry on the methods and standards to be used in law enforcement. The method validation protocols for the establishment of private standards are provided in the guidance of the “International Conference On Harmonisation Of Technical Requirements For Registration Of Pharmaceuticals For Human Use” (ICH) which have been incorporated into the laws and regulations in the European Union, Japan and the USA; these procedures are required for the assessments of new drug entities [2]. The method validation protocols for the establishment of monographs to support public standards are provided in USP <1225> [3] and ICH Q2 [4]. Both protocols cite the same analytical performance characteristics and test procedures except that the public standard must be able to be applied to all legally marketed products containing the specific API whereas the private standard applies only to the approved API in the specific product.

The analytical performance characteristics which must be assessed in both the ICH and USP are Accuracy, Precision (both Repeatability and Intermediate in ICH), and Specificity. Detection Limit, Quantitation Limit, Linearity and Range depending on which attributes are to be assessed. The USP presents the characteristics as noted below [3]:

“Category I — Analytical procedures for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products.

Category II — Analytical procedures for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. These procedures include quantitative assays and limit tests.

Category III — Analytical procedures for determination of performance characteristics (e.g., dissolution, drug release, etc.).

Category IV — Identification tests.”

These can be categorized further into Assay procedures for Category I, Category II, impurity determinations, and Category III (dissolution and drug release are different procedures for preparing a solution of the API), all performance characteristics except Detection Limit must be validated. For the Category II limit tests only the Specificity and Detection Limits must be validated and for Category IV, Identification Tests, only the Specificity needs to be validated. The following sections will provide approaches toward the various aspects of method

validation. Although the approaches are generally applicable to common techniques used in pharmaceutical analysis (such UV-VIS and HPLC quantifications), particular emphasis will be placed on high performance thin layer chromatography (HPTLC) techniques.

2. Specificity

Specificity is the ability of a method to distinguish an analyte from all substances that are present or likely to be present in test samples [3, 4]. When possible, these substances should include future degradation products and other ingredients (i.e., excipients). An analytical procedure is specific when placebo and impurity spots do not overlap partially with and are not buried under the analyte spot. In addition, the calculated amount of analyte does not depend on the quantity of other substances.

Various approaches are possible when evaluating method specificity [5–8]. Ideal demonstration of specificity for an HPTLC analytical procedure requires chromatographing simultaneously three types of samples: sample type 1 is the pure analyte or its reference standard, sample type 2 is the analyte mixed with a representative blank and all likely impurities, and sample type 3 is the representative blank mixed with all likely impurities. Likely impurities include degradation products, reagents, intermediates, excipients, side products, and analyte isomers. The mixtures can be created by spiking test samples (API substances or finished products) or placebos with likely impurities.

In practice, the unavailability of one or more of these types of samples can pose a significant challenge. In some cases, it is often difficult to know all likely impurities. There can be several sample deficiency scenarios. If the pure analyte or its reference standard is not available, demonstration of specificity can be quite challenging if not impossible. If a representative blank is available, but some or all likely impurities are missing, the typical test sample is subjected to stress testing environments. It should be noted however that stress testing is unlikely to produce some analyte isomers, reagents, intermediates and side products. If a representative blank is unavailable, but some likely impurities are available, spiking the typical test sample with impurities can show that increasing impurities will not change analyte signal. In addition, efforts should be made (perhaps by contacting the manufacturer), whenever possible, to create a representative blank even if it's not exactly in the same dosage form as the test sample. If neither a representative blank nor impurity standards are available, the typical test sample is subjected to stress testing to alleviate some of the deficiencies. Once again, the limitations of stress testing should be acknowledged because it may not produce all likely impurities, it may not account for impurities that are completely buried under the analyte signal, and it may not indicate whether some excipients or impurities can react with the analyte.

In general, stress testing, impurity spiking, and peak-purity analysis are the common tools used to address certain sample deficiencies. To demonstrate method specificity, validation reports typically discuss several measures of performance. One measure of specificity is resolution of the analyte spot relative to the closest non-analyte spot. For HPTLC, the resolution should be at least 1 [5].

Analyte peak purity is another measure of specificity that is typically reported. Often, analyte peak purity in the analyte reference standard is compared to analyte peak purity in the other test samples mentioned above. The analysis is performed by comparison of peak spectra at the start, apex, and end of the analyte peak. Some authors use correlation coefficients [9] as a measure of peak purity, and others rely on software algorithms that may involve Matrix Algebra. It should be noted that while peak purity can detect the presence of some impurities in the analyte peak, it does have some limitations. For example, peak-purity analysis does not account for missing impurities that could overlap with the analyte peak, and it does not account for impurities having a spectrum that is similar to that of the analyte. In addition, peak-purity analysis is not applicable for detectors that do not register the entire analyte spectrum for each time point.

A third measure of peak purity is an overlay of chromatograms. This measure is especially useful for showing the analyte peak stability during impurity spiking or stress testing. For example, the chromatograms of a finished pharmaceutical product, before and after accelerated aging, can be overlaid to support method specificity.

3. Accuracy

A succinct definition of accuracy is “nearness to truth”. The ICH guidelines [4] provide the following definition:

“The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.”

In other words, accuracy of a method represents the agreement between an expected value and the value generated by the candidate method (the method value). Therefore, accuracy determination involves determining the expected value, finding the method value and calculating the agreement between the two values [3, 4].

In pharmaceutical testing, accuracy is mainly relevant to quantitative methods, such as assay, content uniformity, dissolution, and impurity quantitation. To determine the accuracy of a quantitative HPTLC method, there are typically four major options, which differ mainly on how the expected value is determined. Unfortunately, the most preferable options are not always feasible due to the non-availability of appropriate reference standards or placebo samples. For each option, we will explain how to determine the expected value and the method value. Agreement between the two values will be addressed later.

3.1. Options for determining the expected value and the method value

3.1.1. Option 1 (using a certified reference standard)

The first option involves using a representative, certified reference standard. We say representative because the certified reference standard needs to have a chemical matrix that is the same as the matrix of a typical unknown sample. So, if the method is intended for API quantitation

in an API substance, the reference standard could contain only the API. However, if the method is intended for finished product testing or for impurity quantitation in API substance, the reference standard should contain the appropriate amount of all the substances typically found in the finished product or API substance. The reference standard should be prepared by an ISO certified reference material manufacturer.

3.1.1.1. Determining the accepted value

If a representative certified reference standard is available, the accepted value is the certified amount of analyte (e.g. API) per given sample of the reference standard.

3.1.1.2. Determining the method value

To obtain the method value, at least nine reference standard solutions are prepared and tested as if they were unknown samples, using the candidate HPTLC method. The average and standard deviations of the results will represent the method value. The standard solutions should contain the following analyte concentrations:

- The first three standard solutions should contain analyte concentrations between 50 and 90% of the analyte's label claim (or quantitation limit for an impurity).
- The next three standard solutions should contain analyte concentrations between 90 and 110% of the analyte's label claim.
- The last three standard solutions should contain analyte concentrations between 110 and 150% of the label.
- Due to the unavailability of representative certified reference standards for most pharmaceutical products, option 1 is rarely used in method validation.

3.1.2. Option 2 (Using a representative blank)

The second option involves using a representative blank, which means a substance or mixture that contains all the chemical components of a typical unknown sample except the analyte. It is important to note that the chemical composition of a representative blank depends on both the analyte and the composition of a typical unknown. For assay, or content uniformity, the representative blank is a placebo. For API quantitation in the API substance, the representative blank is typically the solvent used to dissolve the standard. For impurity quantitation in an API substance, the representative blank is typically the API substance. For impurity quantitation in the finished product, the representative blank is a mixture of the placebo plus all the APIs plus all the typical impurities. During testing, the representative blank should be treated the same way as an unknown sample would be. Care must be taken so that only the absence of analyte distinguishes the representative blank from a typical unknown sample.

3.1.2.1. Determining the accepted value

If a representative blank is available, at least nine samples are prepared by spiking the blank with various amounts of analyte. The accepted value can be represented as the average and

standard deviation of all the amounts of analyte spiked to the representative blank samples. At least nine difference samples should be tested covering a minimum of three different concentrations across the expected range of analyte concentration (50–150% of label claim or impurity limit).

3.1.2.2. Determining the method value

Once the spiked blank samples are prepared, they can be analyzed in parallel using the candidate HPTLC method. The average and standard deviation of the results (expressed in the same unit as the accepted value) can represent the method value.

3.1.3. Option 3 (Using a reference method)

If options 1 and 2 are not feasible, a reference method can be used to determine the accuracy of a candidate method. The reference method must be independent of the candidate method, have been well validated with a stated accuracy, and have the same intended use as the candidate method.

3.1.3.1. Determining the accepted value

To obtain the accepted value for option 3, the reference method can be used to test 6 or more unknown samples. The average and standard deviation of the results will represent the accepted value.

3.1.3.2. Determining the method value

To obtain the method value, each of the samples used to determine the accepted value is tested using the candidate method. The average and standard deviation of the results will represent the method value.

3.1.4. Option 4 (Using standard addition to unknown)

In lieu of option 3, method accuracy can be estimated using the standard addition method [10]. In this case the test sample is an unknown finished product or an API substance, whose analyte amount has been predetermined using the candidate method.

3.1.4.1. Determining the accepted value

To obtain the accepted value for option 4, at least 6 or more stock solutions of unknown samples should be prepared and tested per the candidate method. The average and standard deviation of the results will represent the accepted value.

3.1.4.2. Determining the method value

To obtain the method value, each of the stock solutions used to determine the accepted value is tested once again using the standard addition method [10]. So, each stock solution should have its own standard addition curve with 5 or more data points. The average and standard deviations of the absolute values of the x-intercepts will represent the method value.

3.2. Agreement between expected and method values

Several calculation methods are used to determine the agreement between the expected and method values. The percent recovery method is the simplest. It involves dividing the average method value by the average expected value and multiplying the result by 100. Although this method is considered acceptable in the ICH guidelines [3, 4], and it is found in many publications, it does not take the standard deviations into account.

One method that takes variation into account is the expanded uncertainty interval method [11]. It involves combining the expected and method uncertainties to obtain the expanded uncertainty, which is then compared to the difference between the average expected value and the average method value. If the expanded uncertainty is greater or equal to the difference, the candidate method is considered accurate.

A more statistically rigorous method to calculate accuracy is the t-test for two equal means [12]. It can be performed using MS Excel or other statistical software, but it requires an understanding of hypothesis testing. Interested readers can consult any general Statistics book for more details on t-test and hypothesis testing.

The concept of accuracy profile, which is different from the concept of accuracy described herein, is described by Shewiyo et al. It aims to describe method performance using a single statistic [13].

4. Linearity

Linearity evaluations demonstrate measurements from a test method are proportional to the amount of analyte within a particular concentration range [3, 4]. Responses from samples containing different amounts of analyte are obtained from the test method. Generally, a minimum of five different concentrations should be used where multiple (i.e., ≥ 3) responses are obtained at each analyte level. The method response (y-axis) is plotted as a function of the analyte concentration (x-axis) for subsequent analysis with linear regression techniques, where slope, intercept, and correlation coefficient are reported. The concentration range should cover the upper and lower levels anticipated during an analysis.

In the following example, a graphical representation of a linear calibration model is demonstrated, where the raw data is provided in **Table 1** and **Figure 1** shows the corresponding linear regression curve.

[Concentration], w/v	[Response], Instrument reading (triplicate results)
2	0.06, 0.06, 0.06
4	0.12, 0.12, 0.11
6	0.17, 0.17, 0.16
8	0.22, 0.23, 0.22
10	0.28, 0.28, 0.28

Table 1. Data to demonstrate a linear calibration model.

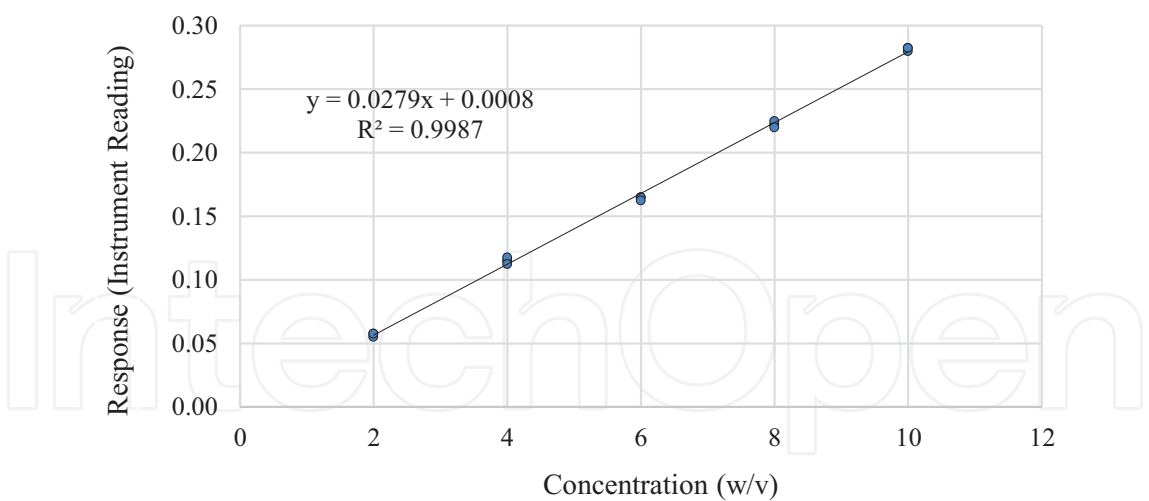


Figure 1. Graphical representation of linear calibration model data.

However, a linear model may not be the best calibration fit for the data as is the case of the data listed in **Table 2**, and plotted in **Figure 2**. When the linear model is applied to the data, the resulting correlation coefficient ($R^2 = 0.98$) is less than ideal.

Further examination of the data indicates that a polynomial fit can provide a better calibration model from the data (**Figure 3**). It should be noted that most pharmaceutical analysis methods commonly use a one-point standard during routine use of the method (after validation has been established).

Variable	Data							
[Concentration], w/v	1	2	3	4	5	6	7	8
[Response], Instrument reading	10	20	30	41	46	55	60	65

Table 2. Data to demonstrate a non-linear calibration model.

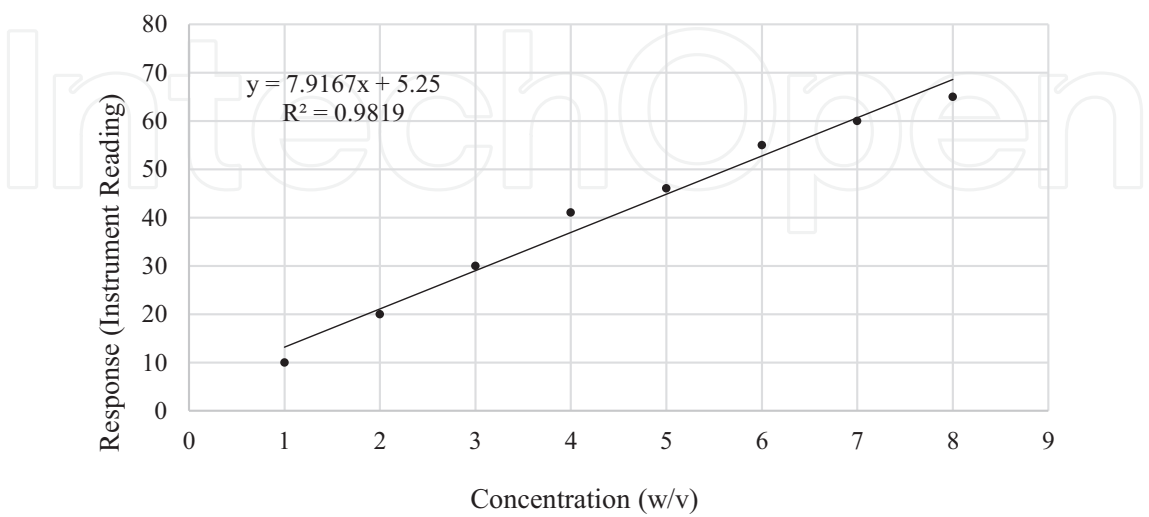


Figure 2. Graphical representation of a less than ideal linear calibration model.

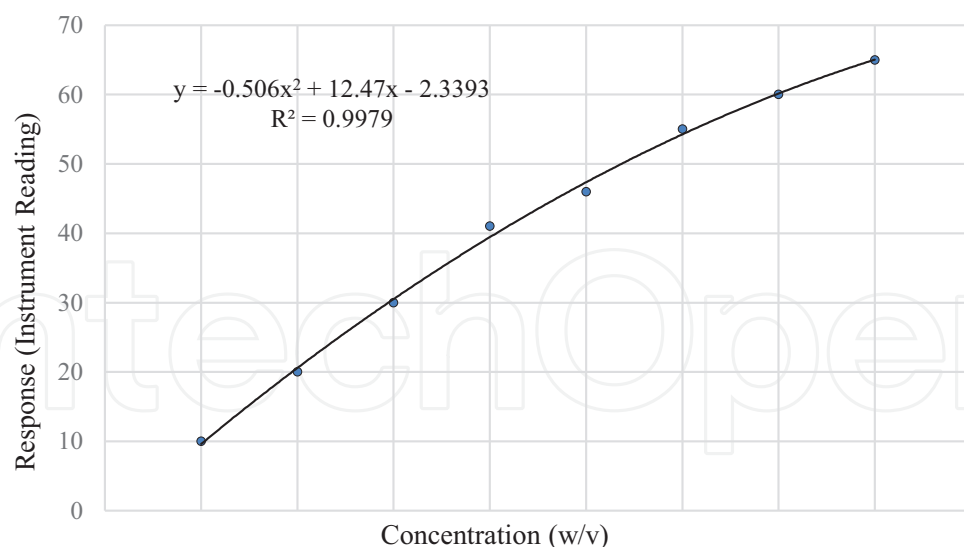


Figure 3. Graphical representation of polynomial approach to calibration model.

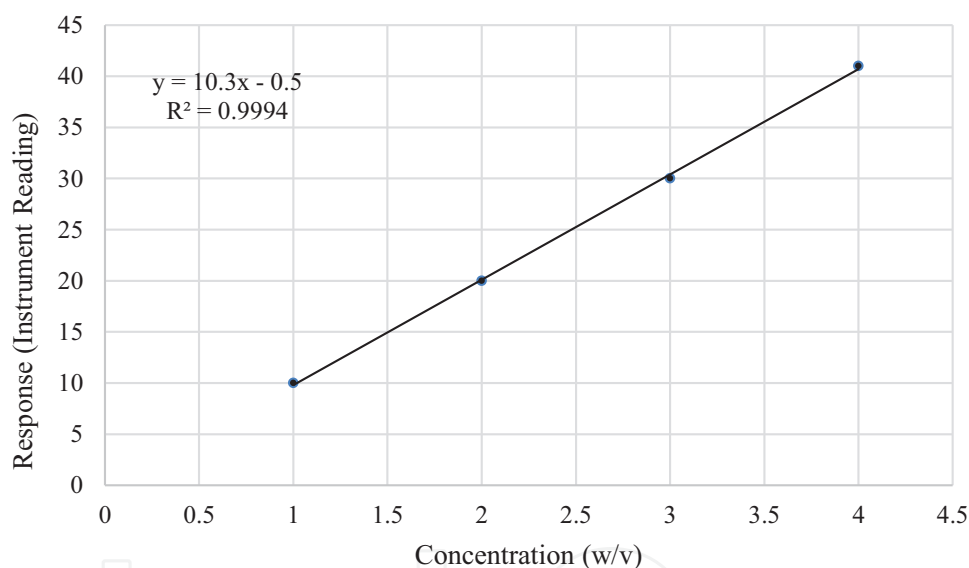


Figure 4. Graphical representation of the more linear range of the preceding polynomial calibration model.

If the analysis range for the method only requires concentrations from 1 to 4 (w/v), a linear model for just that concentration range provides a r^2 of 0.9994 (**Figure 4**) and would be easier to implement in future analysis (note-an additional standard should be added within that range during final validation).

5. Limits of detection/quantitation

Various options are possible for determining limits of detection (LOD) and limits of quantitation (LOQ) [3, 4, 14, 15]. The section below will provide some key example approaches for tests that generate instrument based responses.

The signal to noise ratio can be used to determine both the LOD and LOQ, where responses are obtained from blank and from an array of samples at lower concentrations. A ratio of signal (from analyte samples) to noise (from blank) of 3 is an accepted concentration level for the LOD. Likewise, a concentration level that provides a signal to noise of 10 can be used as the LOQ.

Another approach first involves the determination of the standard deviation of the response and the slope of calibration (linearity) curve. Although other options are possible [3, 4, 14, 15], the standard deviation of the response can be estimated from replicate injections from blank samples or from the standard deviation of y-intercepts from multiple regression lines. Multiplying the ratio of the standard deviation of the responses to the slope of the curve by 3.3 or 10 provides the LOD or LOQ, respectively.

6. Precision

For an analytical method, precision is an assessment of the consistency of results obtained with multiple measurements from the same sample [3, 4, 16]. There are three categories of precision for an analytical method, namely repeatability, intermediate precision, and reproducibility, which can be assessed through variations with different equipment, testing times (conducted on different days), analysts, and/or laboratories.

Repeatability is often evaluated with replicate measurements of a sample on the same day in the same laboratory, where the analyst and equipment are not changed. Intermediate precision can be evaluated from replicate measurements of a sample within the same laboratory, but with systematic variations with different analysts, times of analysis, and equipment (such as different instruments). Reproducibility is commonly determined from replicate measurements of the same sample but within different labs, which will inherently incorporate different analysts, equipment, and time of analysis.

ICH Q2 provides several recommendations for number of replicates and concentration levels for each of the three types of precision [4]. Recommended approaches for repeatability are at least nine measurements that span the method's range (such as three replicates for each of three analyte levels) or at least six measurements at the target analyte level. ICH Q2 does not specify a minimum number of samples for intermediate precision and reproducibility but encourages that the effects of variables (i.e., analysts, days, instruments) be systematically evaluated. The following section will provide possible approaches for evaluating repeatability and intermediate precision, followed by references for examples for reproducibility will be provided.

To perform the appropriate precision assessments, the following equations are indicated [16] and will be used for further development of subsequent examples. The average (\bar{x}) of n replicates is provided in Eq. (1),

$$\bar{x} = \frac{\sum_{i=1}^n x_i}{n} \quad (1)$$

where x_i represents the individual replicates measurements. The standard deviation (s) of a data set can be determined through Eq. (2),

$$s = \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{(n-1)}} \quad (2)$$

and the % relative standard deviation (%RSD) is provided in Eq. (3).

$$\%RSD = 100 \times \left(\frac{s}{\bar{x}}\right) \quad (3)$$

The %RSD is often used in method validation assessments because it normalizes the standard deviation to the average.

From Eqs. (1)–(3), an evaluation of repeatability can be determined. In the following example, assume that an analyst has performed six replicate analysis (within the same laboratory) from a method capable of quantifying the amount of active ingredient in a pharmaceutical product in units of % label claim (assay) and obtained the following results (102.1%, 100.5%, 98.2%, 99.1%, 101.8%, 99.8%). Using Eqs. (1)–(3), the average (\bar{x}), standard deviation (s), and %RSD would be 100.25%, 1.52%, and 1.52%, respectively, where s (or more commonly %RSD), is a measure of method repeatability.

Intermediate precision involves an evaluation of variations “within runs” and “between runs” [17]. Consider the data in **Table 3** containing replicate runs ($n = 3$, indexed with j) obtained on each of multiple days ($p = 5$, indexed with i), where each day used a different analyst with separate solution preparations but using the same method as above for the repeatability analysis.

With each entry in the data representing a separate x_{ij} , the repeatability or within-run standard deviation (days) can be determined using Eq. (4), where \bar{x}_i is provided in Eq. (5).

$$s_r = \sqrt{\frac{\sum_{i=1}^p \sum_{j=1}^n (x_{ij} - \bar{x}_i)^2}{p(n-1)}} \quad (4)$$

$n = 3(j)/p = 5(i)$	Day 1	Day 2	Day 3	Day 4	Day 5
Replicate 1	102.2	98.7	99.3	101.9	102.1
Replicate 2	100.3	101.8	98.1	100.1	101.4
Replicate 3	99.9	102.3	98.7	99.1	101.2
\bar{x}_i	100.8	100.9	98.7	100.4	101.6
$\bar{\bar{x}}$	100.5				

Table 3. Example data for intermediate precision determination.

$$\bar{x}_i = \frac{\sum_{j=1}^n x_{ij}}{n} \quad (5)$$

The between-run standard deviation (days) can be calculated with Eq. (6), where $\bar{\bar{x}}$ is provided in Eq. (7).

$$s_B = \sqrt{\frac{\sum_{i=1}^p (\bar{x}_i - \bar{\bar{x}})^2}{p-1} - \frac{s_r^2}{n}} \quad (6)$$

$$\bar{\bar{x}} = \frac{\sum_{i=1}^p \sum_{j=1}^n x_{ij}}{pn} \quad (7)$$

Subsequently, the intermediate precision standard deviation can be calculated with Eq. (8).

$$s_{IP} = \sqrt{s_r^2 - s_B^2} \quad (8)$$

From the data presented in **Table 3** and using Eqs. (4)–(8) [17], the standard deviations for repeatability (within-run), between-run, and intermediate precision are calculated as 1.26, 0.80, and 1.49, respectively.

Evaluations for reproducibility utilize interlaboratory trials, and are commonly employed when a procedure requires further standardization for use among a more extended array of laboratories. ISO 5725 [18] provides the necessary approach and management structure needed to properly plan, conduct, and interpret the results of an interlaboratory trial that will involve multiple laboratories conducting replicate analysis of a sample(s) at a particular analyte level(s). Approaches are provided to graphically (Mandel's statistics) and quantitatively (Cochran/Grubb) identify outliers so that the most accurate assessments of repeatability and reproducibility variance (standard deviations) are possible. The calculations involved in these types of trials are fairly extensive. Several examples are provided within ISO 5725, and Vander Heyden et al. provides a detailed example for an interlaboratory trial for an HPLC procedure [19].

Overall, desired levels for precision for pharmaceutical analysis are commonly on the order of ~2% RSD. However, different ranges can be necessary depending on the concentration level of the analyte (i.e., higher levels of %RSD can be allowed as the analyte concentration decreases) [17].

7. Robustness

Robustness is a measure of how much a method is impacted by deliberate (small) changes in method conditions [3, 4]. The following are a listing of the types of parameters that can be

evaluated to assess method robustness; solution stability (to heat and or time), extraction conditions during sample preparation (time, temperature, mechanical shaking time, sonication time), type of filters used during final standard/sample preparation, minor adjustments in mobile phase composition, and adjustments in other chromatographic conditions (flow rate, different suppliers of columns, temperature). Commonly, robustness is evaluated during the development stages of the method.

An approach for evaluating robustness could be to compare an analysis using the primary method compared to an analysis where a certain parameter is adjusted. Depending on the method and sample type, adjustments in parameters that generate less than ~2% difference relative to the primary method can provide a reasonable measure of how sensitive the method is to various types of adjustments. During the development of the method for example, a study could be conducted to evaluate the sensitivity of the method on the type of filter by comparing the results from a sample solution that was centrifuged (without filtration) to those filtered with different filter types (PTFE, PVDF, nylon) from different manufacturers. Solution stability could be evaluated by comparing results from freshly prepared solutions compared to the same solutions stored at room temperature over several days.

There are a variety of approaches that can be incorporated to evaluate method robustness. Dejaegher and Vander Heyden provide an extensive review for a variety of approaches to systematically evaluate method robustness (ruggedness) [20].

8. Range

The range of the method corresponds to the lower and upper analyte concentration where satisfactory levels of linearity, precision, and accuracy have been achieved during the method validation process. The range is indicated in the same units as that of the results obtained from the method.

For analysis of pharmaceutical products [3, 4], the following ranges (in percentage relative to the target level) are often required for the respective types of tests; assay (80–120%), content uniformity (approximately 70–130%), impurities (approximately 50–120% of the acceptance limit), dissolution ($\pm 20\%$ of the required range).

9. Application with HPTLC techniques

High-performance thin layer chromatography (HPTLC), an extension of TLC, is a robust, simple, rapid, and efficient tool in quantitative and qualitative analysis of compounds [21], and a variety of applications can be found in the literature [22–29]. In this section an overview of applications of HPTLC in typical pharmaceutical testing protocol is highlighted with examples. HPTLC is one of the sophisticated instrumental techniques based on the full capabilities of thin layer chromatography. The advantages of automation, scanning, full optimization,

selective detection principle, minimum sample preparation, hyphenation, and so on enable it to be a powerful analytical tool for chromatographic information of complex mixtures of pharmaceuticals, natural products, clinical samples, and food stuffs [21]. HPTLC is one of the ideal TLC technique for the analytical purposes because of its increased accuracy, reproducibility, and ability to document the results, compared with standard TLC. Because of this, HPTLC technologies are also the most appropriate TLC technique for conformity with GMPs [30].

9.1. Identification test

In a pharmaceutical testing protocol, identification tests are intended to ensure the identity of an analyte in an API or finished pharmaceutical product sample. This is normally achieved by comparison of a chromatographic behavior of unknown sample to that of a reference standard. The identity of the test substance is confirmed if the migration distance of the test substance matches that of the reference substance. Thin layer chromatography experiments are among the key identity tests in most pharmacopeia monographs. Pharmacopeia standards are typically used by industry as a basis for meeting QC requirements and current good manufacturing practices (cGMPs). Many identification tests in the major pharmacopeia (e.g., USP, Ph. Int., and Ph. Eur. [1, 31, 32]) use planar chromatography (TLC), however HPTLC is a superior technology. **Figure 5** below represent a typical densitogram obtained in the identification of sulfamethoxazole (SMX) and trimethoprim (TPM). In this example, the migration distances are 0.35 and 0.90 for TPM and SMX respectively.

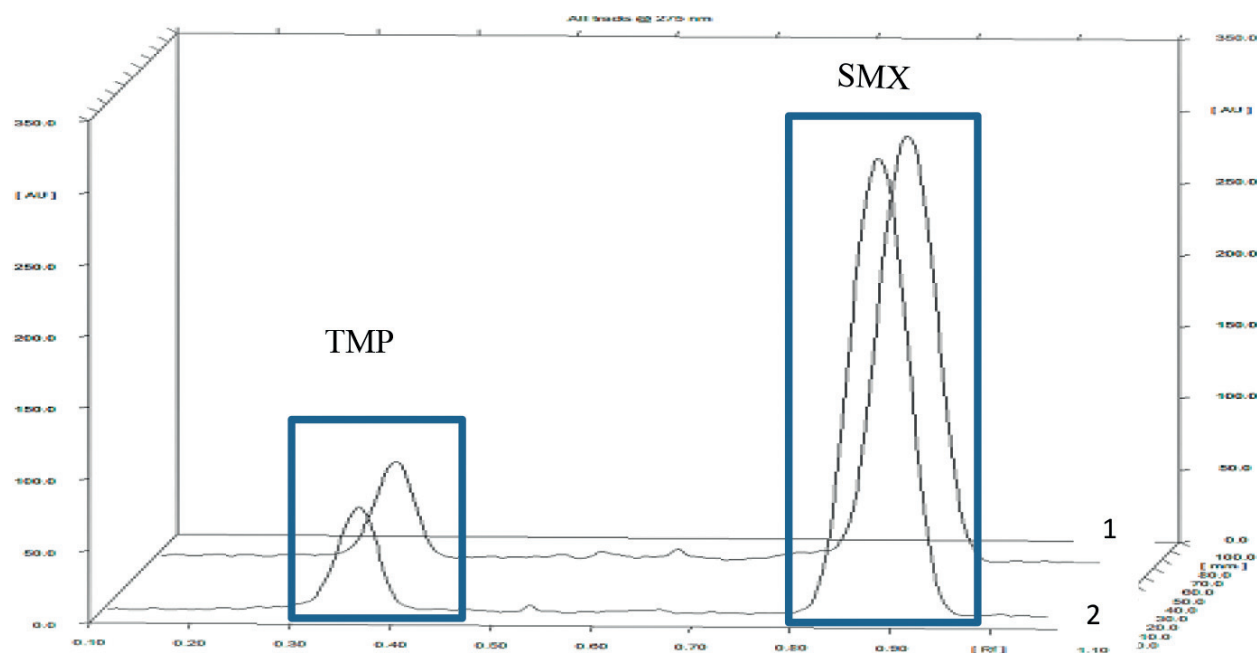


Figure 5. An example of overlaid densitogram for identification of sample 1 and a reference 2 containing sulfamethoxazole (SMX) and trimethoprim (TPM). Conditions Mobile Phase: (Methanol: Ethyl Acetate: Toluene 6: 9:15 v/v) Detection Wavelength: 275 nm and Application Volume: 5 μ l and aluminum plates precoated with silica gel 60 F254 as the stationary phase.

9.2. Assay content determination

A second most important critical quality attribute for pharmaceutical products testing is assay or determination of content. The procedure intended to measure the analyte present in a sample. In this context, the assay represents a quantitative measurement of the major component(s) in the drug substance. This is done by comparing the area under the peak of test substance to that of reference standard material. For a drug product, containing paracetamol an overlaid densitogram is presented in **Figure 6**. Similar validation characteristics also apply when assaying for the active or other selected component(s). The same validation characteristics may also apply to assays associated with other analytical procedures (e.g., dissolution) [33].

9.3. Impurities and related substances

The principal requirement is that an analytical method for assessing impurities should be a stability indicating and meeting specificity criteria described in Section 2 above. Stability indicating method (SIM) is defined as a validated analytical procedure that accurately and precisely measures the active ingredients (drug substance or drug product) free from process impurities, excipients and degradation products. This can be demonstrated by forced degradation study of the drug substance and subjecting the resultant solution to the chromatographic conditions [34].

Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Either test is intended to accurately reflect the purity characteristics of the sample. Quantitative tests for impurities are meant to quantify the exact amount of impurity. This is

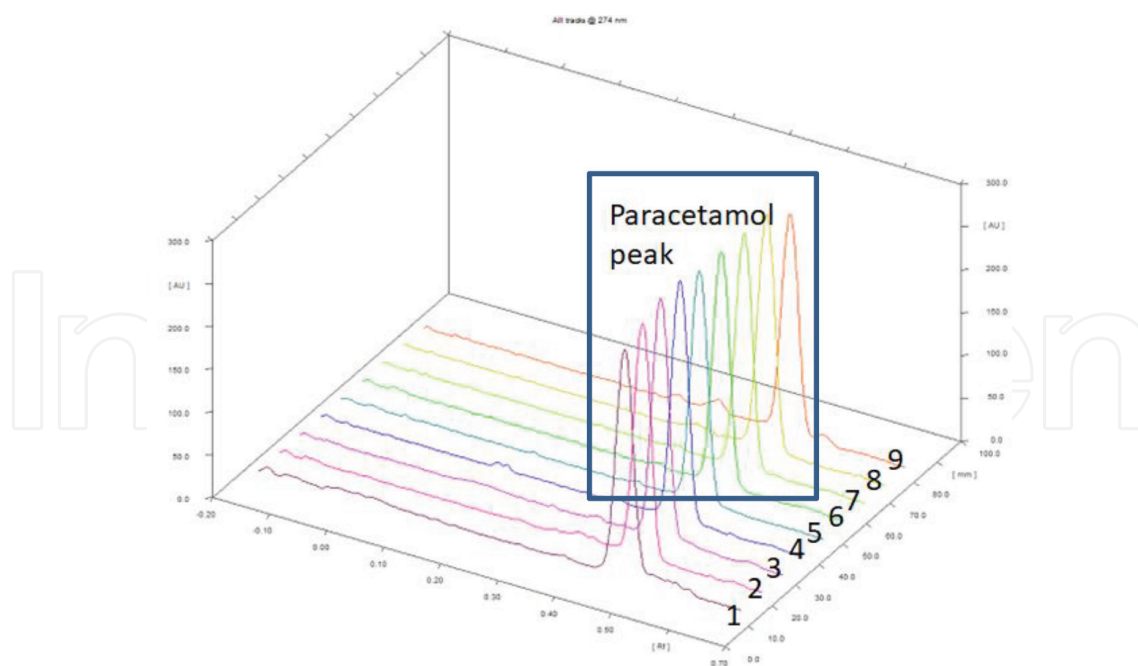


Figure 6. An example of overlaid densitograms for assay of paracetamol in sample tracks 2, 3, 5, 8 and 9, and a reference in tracks 1, 4 and 7. Conditions: Mobile Phase: Acetone; Methanol; Toluene: 6:6:16 v/v/v acidified with three drops of Glacial Acetic Acid, Detection Wavelength: 274 nm, Application Volume: 5 μ l and aluminum plates precoated with silica gel 60 F254 as the stationary phase.

done by comparing the response from a single or multi-level calibration curve. Whereas the limit test is an estimative test where the impurity is controlled not to exceed certain limit. In this case an impurity standard is prepared at the control level and compared to the response from the sample (which should not exceed this level).

With the improved resolution powers of HPTLC (enhanced by reduced particle sizes), it is possible to perform both tests by using HPTLC. In the literature, there are many stability indicating method for various drug substances for example pseudoephedrine and cetirizine in pharmaceutical formulations [35], clopidogrel bisulphate [36] timolol maleate [37] simultaneous determination of ezetimibe and simvastatin [38], piroxicam [39], and estradiol [40].

9.4. Dissolution testing

Dissolution testing is a performance characterizing test and a requirement for all solid oral dosage forms and is used in all phases of development for product release and stability testing [41–43]. It is a key analytical test used for detecting physical changes in an active pharmaceutical ingredient (API) and in the formulated product. It is a multi-unit test and multi-point sampling, making it very tedious. HPTLC offers a multi-channel capability where a total of 18–25 samples can be applied on one plate in form of bands and analyzed simultaneously. One lot of a product will require 6 units (tested in duplicate), plus calibrators (in triplicates at single or multiple levels). HPTLC methods have been successfully deployed for monitoring dissolution profile of diclofenac and acetaminophen [44], and the stability of rifampicin in dissolution medium in presence of isoniazid [45].

9.5. Content uniformity

The test for Content Uniformity (CU) is the assay of the individual content of drug substance(s) in a number of individual dosage units to determine whether the individual contents are within the set limits [46]. Multiple capsules or tablets are selected at random and each are analyzed to determine the active ingredient in each capsule or tablet. The performance efficiency of this method can benefit from the HPTLC multi-channel capabilities. HPTLC has been successful applied in content uniformity of atorvastatin calcium tablets [47], diazepam tablets [48], diosgenin and levodopa [49], nicorandil tablets [50] and rosiglitazone in tablets [51]. All of these HPTLC method examples provide a faster, more cost efficient approach to quantitative testing for routine analysis.

10. Conclusions

Classic method validations for pharmaceuticals involve techniques such as UV-VIS, TLC, and HPLC. This chapter highlights different examples with High Performance Thin Layer Chromatography (HPTLC). General approaches are provided for method validation, as applicable to pharmaceutical assessments, outlined for each of the key aspects (i.e., specificity, accuracy, linearity, limits of detection/quantitation, precision, robustness, and range). Although classical application of pharmaceutical method validation uses techniques such as UV-VIS or HPLC,

important examples are provided using HPTLC techniques that provide high accuracy/precision with minimal use of reagents and other resources.

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References

- [1] USP 40/NF 35, United States Pharmacopeia. 40th ed., National Formulary. 35th ed. Rockville, MD: The United States Pharmacopeia Convention; United Book Press, Inc.; 2017
- [2] International Conference for Harmonization (ICH) [Internet]. 2017. Available from: <http://www.ich.org/home.html> [Accessed: Sep, 25 2017]
- [3] USP <1225> Validation of Compendial Procedures. In: USP 40/NF 35, United States Pharmacopeia. 40th ed., National Formulary. 35th ed. Rockville, MD: The United States Pharmacopeia Convention; United Book Press, Inc.; 2017
- [4] Validation of Analytical Procedures: Text and Methodology Q2(R1) [Internet]. 2005. Available from: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf [Accessed: Sep, 25 2017]
- [5] Ferenczi-Fodor A, Vigh Z, Nagy-Turak A. Validation and quality assurance of planar chromatographic procedures in pharmaceutical analysis. *Journal - Association of Official Analytical Chemists*. 2001;**84**(4):1265-1276
- [6] Maggio RM, Vignaduzzo SE, Kaufman TS. Practical and regulatory considerations for stability-indicating methods for the assay of bulk drugs and drug formulations. *TRAC-Trends in Analytical Chemistry*. 2013;**49**:57-70. DOI: 10.1016/j.trac.2013.05.008
- [7] Bonfilio R, Laignier Cazedey EC, de Araujo MB, Nunes Salgado HR. Analytical validation of quantitative high-performance liquid chromatographic methods in pharmaceutical

- analysis: A practical approach. *Critical Reviews in Analytical Chemistry*. 2012;**42**(1):87-100. DOI: 10.1080/10408347.2012.630926
- [8] Rashmin P, Mrunali P, Nitin D, Nidhi D, Bharat P. HPTLC method development and validation: Strategy to minimize methodological failures. *Journal of Food and Drug Analysis*. 2021;**20**(4):794-804. DOI: 10.6227/jfda.2012200408
- [9] Shaalan R, Belal T, Yazbi F, Elonsy S. Validated HPTLC methods for determination of some selected antihypertensive mixtures in their combined dosage forms. *Bulletin of Faculty of Pharmacy, Cairo University*. 2014;**52**(2):225-237
- [10] Standard-Addition Methods [Internet]. 2012. Available from: https://chemistry.cos.ucf.edu/redesign/wp-content/uploads/2012/10/Overheads-28-34_Spring_14.pdf. [Accessed: Aug, 27 2017]
- [11] Linsinger T. Comparison of a measurement result with the certified value. European Commission - Joint Research Centre Institute for Reference Materials and Measurements (IRMM) [Internet]. 2010. Available from: http://www.erm-crm.org/ERM_products/application_notes/application_note_1/Documents/erm_application_note_1_english_rev3.pdf. [Accessed: Aug, 29 2017]
- [12] Belal T, Mahrous M, Abdel-Khalek M, Daabees H, Khamis M. Validated HPTLC method for the simultaneous determination of alfuzosin, terazosin, prazosin, doxazosin and finasteride in pharmaceutical formulations. *Analytical Chemistry Research*. 2014;**1**:23-31. DOI: 10.1016/j.jep.2015.08.001
- [13] Shewiyo D, Kaale E, Risha P, Dejaegher B, Beer J, Smeyers-Verbeke J, Vander HY. Accuracy profiles assessing the validity for routine use of high-performance thin-layer chromatographic assays for drug formulations. *Journal of Chromatography, A*. 2013;**1293**:159-169. DOI: 10.1016/j.chroma.2013.03.074
- [14] Shrivastava A, Gupta VB. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chron Young Sci*. 2011;**2**:21-25
- [15] Paino T, Moore A. Determination of the LOD and LOQ of an HPLC method using four different techniques. *Pharmaceutical Technology*. Oct. 1999:86-90
- [16] USP <1010> Analytical Data – Interpretation and Treatment. In: USP 40/NF 35, United States Pharmacopeia. 40th ed., National Formulary. 35th ed. Rockville, MD: The United States Pharmacopeia Convention; United Book Press, Inc.; 2017
- [17] Gonzalez A, Herrador M, Asuero A. Intra-laboratory assessment of method accuracy (trueness and precision) by using validation standards. *Talanta*. 2010;**82**:1995-1998. DOI: 10.1016/j.talanta.2010.07.071
- [18] International Organization for Standardization (ISO), Accuracy (trueness and precision) of Measurement Methods and Results – Part 2: Basic Method for the Determination of Repeatability and Reproducibility of a Standard Measurement Method, ISO 5725-2: 1994/Cor.1:2002(E)

- [19] Vander Heyden Y, Saevels J, Roets E, Hoogmartens J, Decolin D, Quaglia M, Van den Bossche W, Leemans R, Smeets O, Van de Vaart F, Mason B, Taylor G, Underberg W, Bult A, Chiap P, Crommen J, De Beer J, Hansen S, Massart D. Interlaboratory studies on two high-performance liquid chromatographic assays for tylosin (tartrate). *Journal of Chromatography, A*. 1999;**830**:3-28. DOI: 10.1016/S0021-9673(98)00840-1
- [20] Dejaegher B, Vander Heyden Y. Ruggedness and robustness testing. *Journal of Chromatography, A*. 2007;**1158**:138-157. DOI: 10.1016/j.chroma.2007.02.086
- [21] Attimarad M, Ahmed KKM, Aldhubaib BE, Harsha S. High-performance thin layer chromatography: A powerful analytical technique in pharmaceutical drug discovery. *Pharmaceutical Methods*. 2011;**2**(2):71-75. DOI: 10.4103/2229-4708.84436
- [22] Abourashed EA, Mossa JS. HPTLC determination of caffeine in stimulant herbal products and power drinks. *Journal of Pharmaceutical and Biomedical Analysis*. 2004;**36**(3): 617-620. DOI: 10.1016/j.jpba.2004.06.029
- [23] Ansari MJ, Ahmad S, Kohli K, Ali J, Khar RK. Stability-indicating HPTLC determination of curcumin in bulk drug and pharmaceutical formulations. *Journal of Pharmaceutical and Biomedical Analysis*. 2005;**39**(1-2):132-138. DOI: 10.1016/j.jpba.2005.03.021
- [24] Dar AA, Sangwan PL, Khan I, Gupta N, Qaudri A, Tasduq SA, et al. Simultaneous quantification of eight bioactive secondary metabolites from *Codonopsis ovata* by validated high performance thin layer chromatography and their antioxidant profile. *Journal of Pharmaceutical and Biomedical Analysis*. 2014;**100**:300-308. DOI: 10.1016/j.jpba.2005.03.021
- [25] Kaul N, Dhaneshwar SR, Agrawal H, Kakad A, Patil B. Application of HPLC and HPTLC for the simultaneous determination of tizanidine and rofecoxib in pharmaceutical dosage form. *Journal of Pharmaceutical and Biomedical Analysis*. 2005;**37**(1):27-38. DOI: 10.1016/j.jpba.2004.09.036
- [26] Koba M, Marszall M, Sroka W, Tarczykowska M, Bucinski A. Application of HPTLC and LC-MS methods for determination of topiramate in pharmaceutical formulations. *Current Pharmaceutical Analysis*. 2012;**8**(1):44-48
- [27] Mahgoub H, Youssef RM, Korany MA, Khamis EF, Kamal MF. Development and validation of spectrophotometric and HPTLC methods for simultaneous determination of rosiglitazone maleate and metformin hydrochloride in the presence of interfering matrix excipients. *Drug Development and Industrial Pharmacy*. 2014;**40**(9):1190-1198. DOI: 10.3109/03639045.2013.810634
- [28] Minz S, Kaurav M, Sahu KK, Mandal V, Pandey RS. Development and validation of TLC-densitometric method for determination of lipid A adjuvant as a bulk and in solid fat nanoemulsions. *Biomedical Chromatography*. 2015;**29**(10):1473-1479. DOI: 10.1002/bmc.3444
- [29] Shirode AR, Ghuge AD, Kadam VJ. HPTLC method development and validation of cinnarizine in bulk and marketed formulation. *International Journal of Pharmaceutical Sciences and Research*. 2016;**7**(6):2416-2422. DOI: 10.13040/ijpsr.0975-8232.7(6).2416-22

- [30] Sudberg S, Sudberg EM, Terrazas J, Sudberg S, Patel K, Pineda J, et al. Fingerprint analysis and the application of HPTLC to the determination of identity and quality of botanicals, from an industry perspective. *Journal of AOAC International*. 2010;**93**(5):1367-1375
- [31] World Health Organization. The International Pharmacopoeia. 6th ed. Geneva: World Health Organization, Dept. of Essential Medicines and Pharmaceutical Policies [Internet]. 2008. Available from: <http://apps.who.int/phint/en/p/about/>. [Accessed: Sep, 26 2017]
- [32] The Council of Europe. European Pharmacopoeia 8.0: Strasbourg, France; 2014
- [33] Shewiyo D, Kaale E, Risha P, Dejaegher B, Smeyers-Verbeke J, Vander HY. HPTLC methods to assay active ingredients in pharmaceutical formulations: A review of the method development and validation steps. *Journal of Pharmaceutical and Biomedical Analysis*. 2012;**66**:11-23. DOI: 10.1016/j.jpba.2012.03.034
- [34] Farid NF, Abdelwahab NS. Stability-indicating HPTLC method for studying stress degradation behavior of sulbutiamine HCl. *Journal of Chromatographic Science*. 2016;**54**(4): 609-617. DOI: 10.1093/chromsci/bmv226
- [35] Makhija SN, Vavia PR. Stability indicating HPTLC method for the simultaneous determination of pseudoephedrine and cetirizine in pharmaceutical formulations. *Journal of Pharmaceutical and Biomedical Analysis*. 2001;**25**(3):663-667. DOI: 10.1016/S0731-7085(00)00586-0
- [36] Agrawal H, Kaul N, Paradkar A, Mahadik K. Stability indicating HPTLC determination of clopidogrel bisulphate as bulk drug and in pharmaceutical dosage form. *Talanta*. 2003;**61**(5):581-589. DOI: 10.1016/S0039-9140(03)00364-3
- [37] Kulkarni SP, Amin PD. Stability indicating HPTLC determination of timolol maleate as bulk drug and in pharmaceutical preparations. *Journal of Pharmaceutical and Biomedical Analysis*. 2000;**23**(6):983-987. DOI: 10.1016/S0731-7085(00)00389-7
- [38] Dixit RP, Barhate CR, Nagarsenker MS. Stability-indicating HPTLC method for simultaneous determination of ezetimibe and simvastatin. *Chromatographia*. 2008;**67**(1-2):101-107. DOI: 10.1365/s10337-007-0459-4
- [39] Puthli S, Vavia P. Stability indicating HPTLC determination of piroxicam. *Journal of Pharmaceutical and Biomedical Analysis*. 2000;**22**(4):673-677. DOI: 10.1016/S0731-7085(99)00300-3
- [40] Kotiyan P, Vavia P. Stability indicating HPTLC method for the estimation of estradiol. *Journal of Pharmaceutical and Biomedical Analysis*. 2000;**22**(4):667-671. DOI: 10.1016/S0731-7085(99)00301-5
- [41] USP <711> Dissolution. In: USP 40/NF 35, United States Pharmacopeia. 40th ed., National Formulary. 35th ed. Rockville, MD: The United States Pharmacopeia Convention; United Book Press, Inc.; 2017
- [42] Zhang H, Yu L. Dissolution testing for solid oral drug products: Theoretical considerations. *American Pharmaceutical Review*. 2004;**7**(5):26-31. DOI: 10.1023/A:1011984216775

- [43] U.S. FDA/CDER. Guidance for Industry: Dissolution testing of immediate release solid oral dosage forms [Internet]. 1997. Available from: <https://www.fda.gov/downloads/drugs/guidances/ucm070237.pdf> [Accessed: Sep, 26 2017]
- [44] Shah H, Rathod I, Shah S, Savale S, Shishoo C. Sensitive HPTLC method for monitoring dissolution profiles of diclofenac from different tablets containing combined diclofenac and acetaminophen. JPC Journal of Planar Chromatography Modern TLC. 2003 Feb 1; **16**(1):36-44. DOI: 10.1556/JPC.16.2003.1.8
- [45] Shishoo C, Shah S, Rathod I, Savale S, Kotecha J, Shah P. Stability of rifampicin in dissolution medium in presence of isoniazid. International Journal of Pharmaceutics. 1999;**190**(1):109-123. DOI: 10.1016/S0378-5173(99)00286-0
- [46] USP <905> Uniformity of Dosage Units In: USP 40/NF 35, United States Pharmacopeia. 40th ed., National Formulary. 35th ed. Rockville, MD: The United States Pharmacopeia Convention; United Book Press, Inc.; 2017
- [47] Yadav SS, Mhaske DV, Kakad A, Patil B, Kadam S, Dhaneshwar S. A simple and sensitive HPTLC for the determination of content uniformity of atorvastatin calcium tablets. Indian Journal of Pharmaceutical Sciences. 2005;**67**(2):182
- [48] Machale V, Gatade A, Sane R. Validated HPTLC content uniformity test for the determination of diazepam in tablet dosage form. International Journal of Research and Development. 2011;**3**:34-41
- [49] Kshirsagar VB, Deokate UA, Bharkad VB, Khadabadi SS. HPTLC method development and validation for the simultaneous estimation of diosgenin and levodopa in marketed formulation. Asian Journal of Research in Chemistry. 2008 Jul;**1**(1):36-39
- [50] Patel CN, Shah SA, Rathod IS, Savale SS. A simple and sensitive HPTLC method for the determination of content uniformity of nicorandil tablets. Indian Journal of Pharmaceutical Sciences. 2002;**64**(4):362
- [51] Walode SG, Chaudhari HK, Saraswat MS, Kasture AV, Wadodkar SG. Validated high performance thin layer chromatographic determination and content uniformity test for rosiglitazone in tablets. Indian Journal of Pharmaceutical Sciences. 2010 Mar;**72**(2):249

